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Serum angiopoietin-2 and soluble VEGF receptor 2 are surrogate markers for plasma leakage in patients with acute dengue virus infection

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ABSTRACT

Background: Endothelial cell dysfunction is believed to play an important role in the pathogenesis of plasma leakage in patients with acute dengue virus (DENV) infection. Several factors, produced by activated endothelial cells, have been associated with plasma leakage or severe disease in patients with infectious diseases.
Objectives: The aim of this study was to investigate which of these markers could serve as a surrogate marker for the occurrence of plasma leakage in patients with acute DENV infection.
Study design: A case-control study was performed in patients with acute DENV infection in Santos, Brazil. Plasma leakage was detected with X-ray and/or ultrasound examination at admission. Serum levels of soluble endoglin, endothelin-1, angiopoietin-2, VEGF, soluble VEGFR-2, MMP-2, MMP-9, TIMP-1 and TIMP-2 were determined using commercially available ELISAs.
Results: Increased levels of angiopoietin-2, endothelin-1 and MMP-2 and decreased levels of soluble VEGFR-2 were significantly associated with the occurrence of plasma leakage. An unsupervised cluster analysis confirmed that angiopoietin-2 and soluble VEGFR-2 were strongly associated with clinical apparent vascular leakage.
Conclusion: Angiopoietin-2 and soluble VEGFR-2 can serve as surrogate markers for the occurrence of plasma leakage in patients with acute DENV infection.

1. Background

Dengue virus (DENV) is a flavivirus, which is transmitted by the bite of a mosquito. A recent study showed that 390 million persons are infected with DENV each year, of which 96 million develop clinical symptoms [1]. A hallmark of dengue disease is an increase in vascular permeability, presented as pleural effusion and/or ascitis. In severe cases, extensive plasma leakage may lead to the development of hypotension and shock [2].

Endothelial cells play a crucial role in the development of plasma leakage during DENV infection. DENV can infect endothelial cells

Abbreviations: DENV, dengue virus; VEGF, vascular endothelial growth factor; MMP, matrix metalloproteinase; TIMP, tissue inhibitor of matrix metalloproteinases; Ang, angiopoietin; Eng, endoglin; ET-1, endothelin-1; RT-PCR, real time PCR; Chi, Chi-squared test; MWU, Mann–Whitney *U* test; F, Fisher's exact test; MV, missing value; WS−, non-severe dengue without warning signs; WS+, non-severe dengue with warning signs; HC, healthy control.

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Table 1
Characteristics of patients with and without plasma leakage.

	No plasma leakage ($N=56$)	Plasma leakage ($N=49$)	Healthy controls ($N=15$)	Significance
Sex	59% male ($N=33$)	63% male ($N=31$)	60% male ($N=9$)	$p=0.9$ (Chi)
Age*	21 (11–45)	12 (8–29)	25 (24–28)	$p=0.001$ (KW)
Day of fever*	5 (4–6)	5 (4–7) MV = 1	NA	$p=0.9$ (MWU)
2009 WHO dengue case classification	64% ($N=36$) WS− 34% ($N=19$) WS+ 2% ($N=1$) Severe	100% ($N=49$) WS+	NA	$p<0.0001$ (Chi)
Admission	52% ($N=29$) MV=2	96% ($N=47$) MV=1	NA	$p<0.0001$ (F)
Type plasma leakage	–	Ascites: 29% ($N=14$) Pleural: 16% ($N=8$) Pleural and pericardium: 55% ($N=27$)	NA	
Haemorrhagic manifestations	30% ($N=17$)	47% ($N=23$)	NA	$p=0.1$ (F)
Type haemorrhagic manifestation	70% ($N=39$) No 25% ($N=14$) Minor mucosal 5% ($N=3$) Petechiae	53% ($N=26$) No 31% ($N=15$) Minor mucosal 16% ($N=8$) Petechiae	NA	
Platelet count*	122.500 (57.250–166.500) MV = 2	42.000 (33.000–73.000)	NA	$p<0.0001$ (MWU)
Viremic	73% ($N=41$)	59% ($N=29$)	NA	$p=0.2$ (F)
Viral copy number in viremic patients (copies/ml)*	198 (122–950)	126 (100–256)	NA	$p=0.01$ (MWU)
IgG avidity*	21% ($N=12$) Not detectable 2% ($N=1$) Primary 77% ($N=43$) Secondary	2% ($N=1$) Not detectable 98% ($N=48$) Secondary	NA	$p=0.006$ (Chi)

Abbreviations: Statistical test used is the: Chi = Chi-squared test; KW = Kruskal Wallis test; MWU = Mann–Whitney *U* test; F = Fisher's exact test. MV = missing value. WS− = non-severe dengue without warning signs. WS+ = non-severe dengue with warning signs. NA = not applicable.

* Values are in median (interquartile range).

in vitro, but whether this also occurs in vivo, is still a matter of debate [3,4]. Moreover, it is not clear whether DENV causes vascular permeability by direct infection of endothelial cells or through the release of vasoactive agents by infected monocytes and macrophages, which are the primary target cells of DENV infection [4]. In vitro, direct infection of endothelial cells did not lead to an increase in permeability, while co-incubation of endothelial cells with mononuclear cells or the supernatant from DENV-infected monocytic cells did result in an increase [5,6]. This suggests that mechanisms other than direct infection may activate endothelial cells, resulting in an increase in vascular permeability. It is believed that uncontrolled endothelial activation and subsequent dysfunction contributes to the severity of dengue (reviewed in [7]).

Vascular endothelial growth factor (VEGF), initially identified as vascular permeability factor, promotes the growth, proliferation and migration of endothelial cells. VEGF is increased in DENV infected patients with plasma leakage, especially around the time of defervescence [8,9]. VEGF can be bound to sVEGFR-1 and sVEGFR-2, which are expressed predominantly on endothelial cells [10]. Levels of sVEGFR-1 were increased in patients with severe dengue, contrasting with decreased levels of sVEGFR-2 [8].

Matrix metalloproteinases (MMPs) are proteolytic enzymes that can cleave proteins of the extracellular matrix [11]. The activity of these enzymes is regulated by tissue inhibitors of matrix metalloproteinases (TIMPs). Endothelial cells produce MMP-2 and MMP-9 and also TIMP-1 and TIMP-2 [12]. Increased levels of MMP-9 were detected in patients with severe DF compared to mild DF [13]. In the same study, no significant differences were detected in MMP-2 levels between dengue fever patients and healthy controls.

Angiopoietin-1 (Ang-1) is produced by perivascular cells and has a stabilizing effect on the vascular barrier [14]. Angiopoietin-2 (Ang-2) is synthesized by endothelial cells and is a potent inducer of vascular permeability by counteracting the barrier stabilizing effects of Ang-1 [15]. Decreased levels of Ang-1 and increased levels of Ang-2 were correlated with the occurrence of plasma leakage in DENV infected patients, suggesting that an imbalance between these two proteins may be involved in endothelial dysfunction [16].

Activated endothelial cells produce a number of other proteins, including soluble endoglin (sEng) and endothelin-1 (ET-1). Upon inflammation, Eng is cleaved by MMPs and released in the circulation as sEng. sEng binds to TGF-β1 and abrogates its anti-inflammatory effects. Levels of sEng were increased in children with severe malaria [17]. ET-1 is produced by endothelial cells and is a potent vasoconstrictor and has inotropic, chemotactic and mitogenic properties [18]. Increased levels have been detected in patients with sepsis and malaria [19,20].

2. Objectives

The aim of this study was to investigate which of the following markers sEng, ET-1, MMP-2, MMP-9, TIMP-1, TIMP-2, Ang-2, VEGF and sVEGFR-2, all produced by activated endothelial cells, could serve as a surrogate marker for the increase in vascular permeability during DENV infection.

3. Study design

3.1. Clinical cohort

This cohort has been previously described [21–24]. Briefly, during the 2010 outbreak, samples were collected from patients with clinical suspected dengue presenting at the Ana Costa

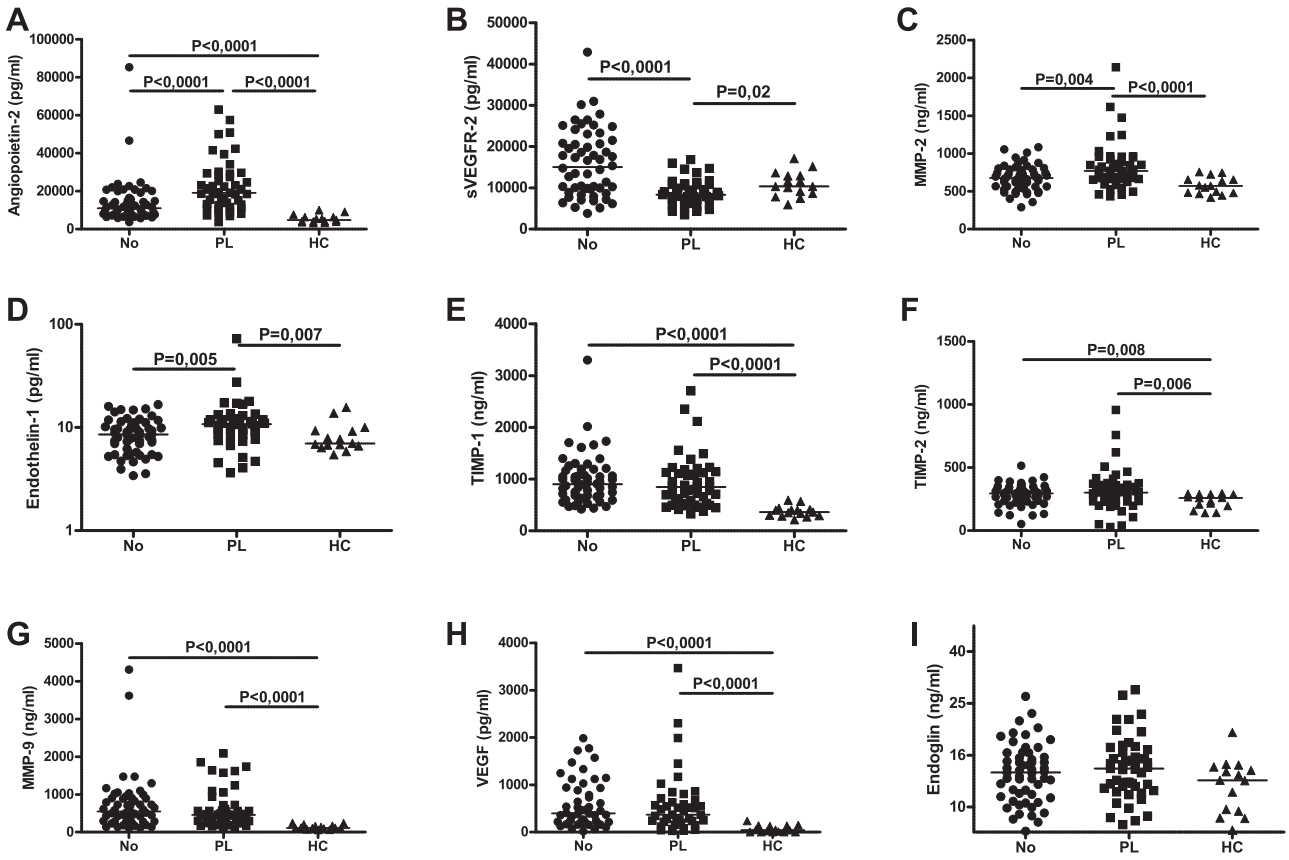

Fig. 1. The association of endothelial markers with the occurrence of plasma leakage. Levels of Ang-2 (A), MMP-2 (C) and ET-1 (D) were significantly increased and levels of sVEGFR-2 (B) were significantly decreased in patients with plasma leakage compared to patients with no plasma leakage. Levels of Ang-2 (A), TIMP-1 (E), TIMP-2 (F), MMP-9 (G) and VEGF (H) were significantly elevated in dengue patients compared to healthy controls. Levels of sEng (I) were not significantly different in any of the groups. *Abbreviations*: No, no plasma leakage; PL, plasma leakage; HC, healthy control. Missing values: Ang-2, sVEGFR-2: PL ($N = 2$); MMP-2, ET-1, MMP-9: PL ($N = 1$); TIMP-1, TIMP-2, sEng: no missing values; VEGF: No ($N = 4$), PL ($N = 5$).

Hospital, Santos, State of São Paulo. Patients were diagnosed with DENV infection by detection of DENV NS1 antigen and/or IgM-specific antibodies using a commercially available rapid test (Dengue duo test bioeasy, Standard Diagnostic Inc., 575-34, Korea) or by detection of DENV RNA by real time PCR (RT-PCR). Serum samples were drawn and stored at −80 °C. Patients were classified according to the 2009 WHO classification [25,26] and the occurrence of plasma leakage. The occurrence of plasma leakage was detected by ultrasound or X-ray examination performed upon a clinical suspicion for plasma leakage, such as hemoconcentration, profound thrombocytopenia, tachycardia, hypotension or dehydration. Healthy volunteers with a similar socio-economic background were used as the reference group.

3.2. IgG avidity ELISA

The IgG avidity test was used to determine primary or secondary DENV infection [27]. Samples with low avidity IgG antibodies were classified as primary DENV infection, whereas samples with high avidity IgG antibodies were classified as secondary. Samples in which IgG antibodies were not detected could not be classified, although the majority was probably primary DENV infection.

3.3. Determination of viral load

Viral load was determined by an "in-house" RT-PCR method. This method has been previously described in detail [24]. RNA was extracted from plasma using the Qiagen Viral RNA kit (Qiagen, Germany). The RT-PCR was conducted in duplicate. SuperScript III Platinum SYBR Green One-Step qRT-PCR kit with ROX (Invitrogen, Inc., EUA) was used. Primers covering all four DENV serotypes were used of which the sequences have been published [28].

3.4. Endothelial cell markers

Levels of sEng, ET-1, MMP-2, MMP-9, TIMP-1, TIMP-2, Ang-2, VEGF and sVEGFR-2 were measured using commercially available ELISA kits ('Quantikine', R&D systems, USA). The sensitivity limits in the diluted samples were sEng (0.03 ng/ml), ET-1 (0.207 pg/ml), MMP-2 (0.082 ng/ml), MMP-9 (0.156 ng/ml), TIMP-1 (0.08 ng/ml), TIMP-2 (0.064 ng/ml), Ang-2 (21.3 pg/ml), VEGF (9 pg/ml) and sVEGFR-2 (11.4 pg/ml). The assays were performed according to the manufacturer's instructions. Every sample was run in duplicates. Repetitive freeze–thaw cycles were avoided.

3.5. Cluster analysis

The cluster analysis procedure was adapted from van den Ham et al. [29]. Briefly, permeability marker values were log-transformed and subjected to hierarchical correlation clustering (i.e., with distance measure 1 – pearson's pairwise correlation value) using Ward's method that minimizes within-cluster variance. Both patients and permeability markers were clustered to obtain a heatmap. VEGF was excluded and one patient as well, because too many values were missing. Cluster analysis was performed in R 2.15 (R Development Core Team [R Foundation for Statistical Computing], 2012, www.r-project.org). R scripts used to construct the trees and heatmaps are available upon request.

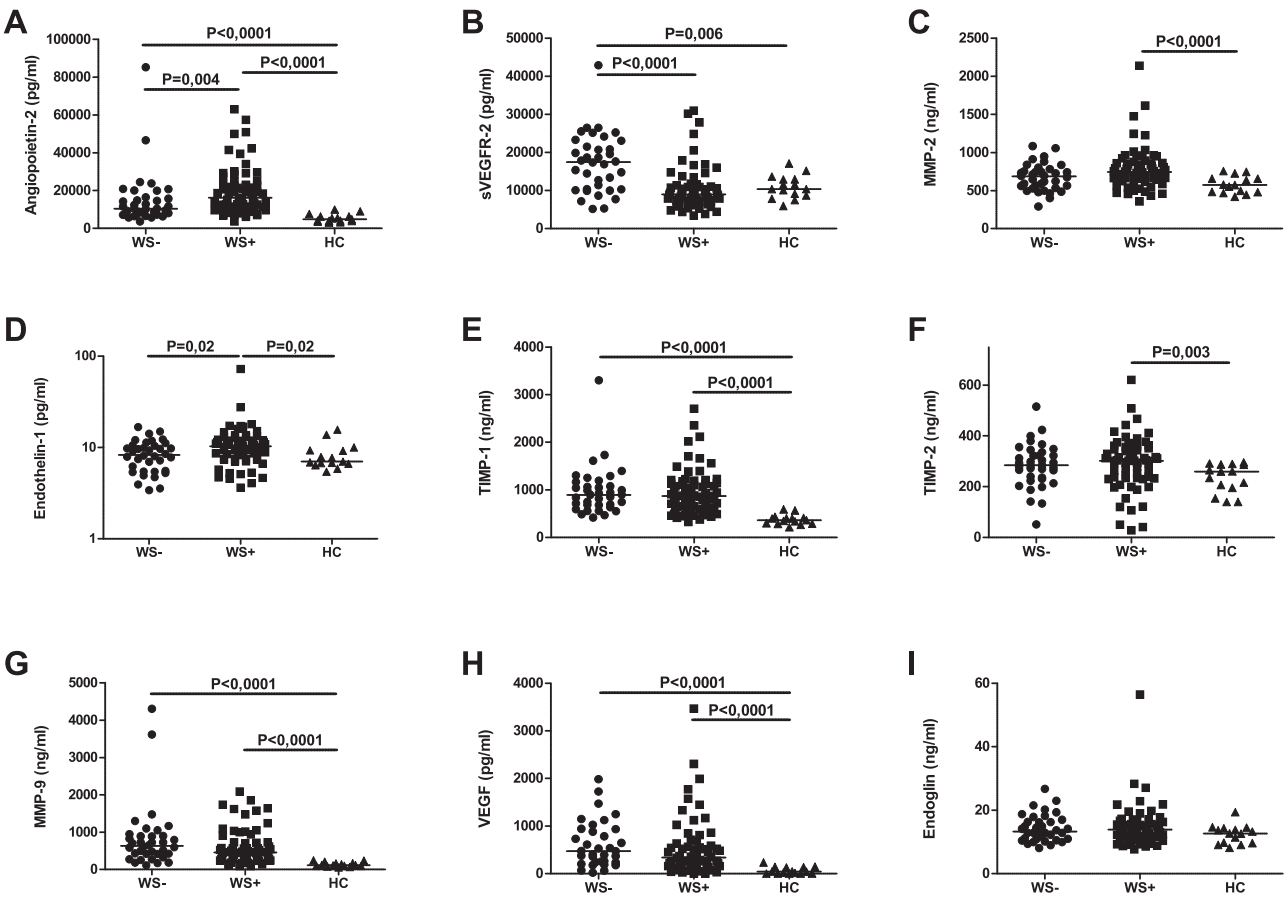

Fig. 2. The association of endothelial markers with the 2009 WHO dengue case classification. Levels of Ang-2 (A) and ET-1 (D) were significantly increased and levels of sVEGFR-2 (B) were significantly decreased in patients with WS+ compared to patients with WS− dengue. Levels of Ang-2 (A), TIMP-1 (E), MMP-9 (G) and VEGF (H) were significantly elevated in dengue patients compared to healthy controls. Levels of MMP-2 (C) and TIMP-2 (F) were significantly increased in WS+ compared to healthy controls. Levels of sEng (I) were not significantly different in any of the groups. *Abbreviations*: WS−, non-severe dengue without warning signs; WS+, non-severe dengue with warning signs; HC, healthy control. Missing values: Ang-2, sVEGFR-2: WS+ ($N=2$); MMP-2, ET-1, MMP-9: WS+ ($N=1$); TIMP-1, TIMP-2, sEng: no missing values; VEGF: WS− ($N=2$), WS+ ($N=7$).

3.6. Statistical analysis

The Kruskal Wallis *H* and the Mann Whitney *U* test were used for comparisons of more than two or between two groups, respectively. Correlations were determined using the Spearman's correlation coefficient. The Chi-squared test was used to calculate whether the distribution was significantly different between more than two groups and/or conditions and the Fisher's exact test in case of two groups/conditions. *P* values ≤ 0.05 were considered significant. Using the Bonferroni correction, a *p*-value cut-off of ≤ 0.02 for endothelial cell marker analyses was applied.

4. Results

For this study, 105 out of 811 patients with laboratory confirmed acute DENV infection were selected based on the absence or presence of plasma leakage and the availability of sample and clinical data. Forty-nine patients with plasma leakage and fifty-six without were included and they were stratified based on gender and day after the onset of fever. Patients with severe co-morbidity were excluded from this analysis. The clinical characteristics of the patients are depicted in Table 1.

Patients with plasma leakage were significantly younger ($p=0.001$), had significantly lower levels of platelets ($p<0.0001$) and a lower viral load ($p=0.01$). Patients with plasma leakage were also less often viraemic, although this difference was not significant. The IgG avidity test indicated that 98% of patients with plasma leakage suffered from a secondary DENV infection. Of the patients without plasma leakage, 21% had undetectable IgG antibodies, suggesting that these patients presented with their first DENV episode. When patients were classified according to the 2009 WHO classification 69% from the WS− and 96% from the WS+ patients suffered from a secondary DENV infection.

To determine the association of endothelial cell markers with the occurrence of plasma leakage, we have measured the levels of nine endothelial cell markers in the serum of DENV infected patients and healthy controls. Levels of Ang-2, MMP-2 and ET-1 were significantly increased and levels of sVEGFR-2 significantly decreased in patients with plasma leakage compared to patients without plasma leakage (Fig. 1). The other markers, TIMP-1, TIMP-2, MMP-9 and VEGF, were significantly elevated in DENV infected patients compared to healthy controls. Levels of sEng were not significantly different in any of the groups. The expression of Ang-2, MMP-2, ET-1 and sVEGFR-2 was only associated with the occurrence, but not with the type of plasma leakage (data not shown).

When patients were classified according to the 2009 WHO dengue case classification, levels of Ang-2 and ET-1 were also significantly elevated and levels of sVEGFR-2 significantly decreased in patients with WS+ compared to WS− dengue (Fig. 2).

To investigate whether time after the onset of disease had an impact on the expression of these nine markers, patients were divided into three groups consisting of 2–3 days, 4–6 days and $\geq$7 days after onset of symptoms (Supplementary Fig. 1). There

were no significant differences between the groups, suggesting that time after the onset of disease, independent from the occurrence of plasma leakage, did not have a major impact on the expression of these markers. Moreover, the Spearman's correlation coefficient was also not significant when the endothelial markers were directly correlated with the day after the onset of disease (data not shown).

The cluster analysis groups patients based on similarities in the expression of the determined markers (Fig. 3). In the resulting heatmap, a dendrogram shows the similarity between the subjects (left side of Fig. 3), where subjects in the same branch are more similar to each other than to subjects in other branches. The subject dendrogram was divided into three main clusters of which cluster A contained the largest proportion of DENV infected patients with plasma leakage and cluster B contained the largest proportion of healthy controls (Table 2). Levels of sVEGFR-2 were clearly decreased and levels of Ang-2 increased in cluster A. Plasma leakage occurred significantly more often in cluster A than the other clusters (Chi-squared test, $p < 0.0001$). Levels of MMP-9 were increased in cluster C compared to the other clusters.

The Spearman's correlation coefficient was used to correlate the endothelial permeability markers with the platelet count and with each other. Ang-2 and sVEGFR-2 correlated strongly with the platelet count (Ang-2: $\rho = -0.63$ ($p < 0.0001$), sVEGFR-2 $\rho = 0.74$ ($p < 0.0001$)) (Fig. 4A and B). Ang-2 and sVEGFR-2 also correlated strongly with each other ($\rho = -0.52$ ($p < 0.0001$)) (Fig. 4C). Moreover, a strong correlation was found between MMP-2 and TIMP-2 ($\rho = 0.60$ ($p < 0.0001$)) (Fig. 4D). Interestingly, TIMP-2 has been described to play a central role in modulating MMP-2 activity [30].

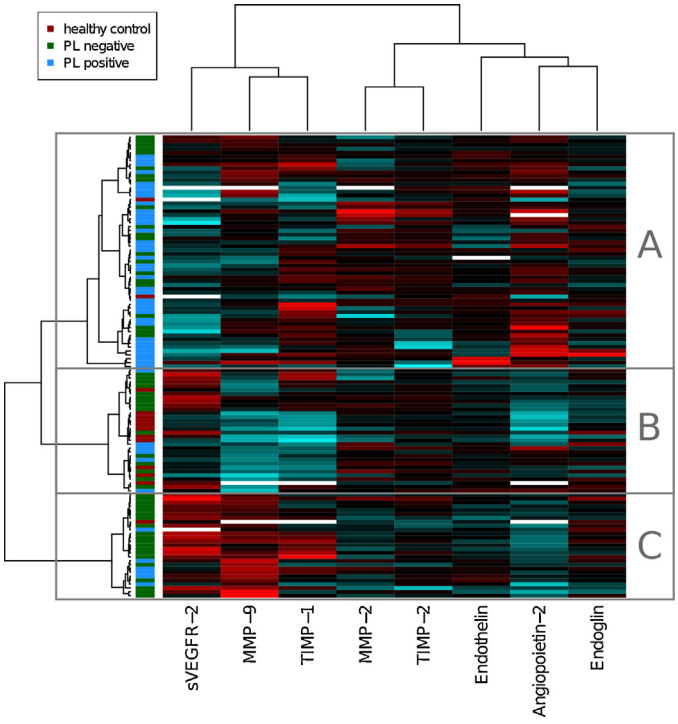

Fig. 3. Heatmap of the cluster analysis. A cluster analysis was performed with eight endothelial markers, which resulted in a dendrogram indicated on the left of the heatmap. Every horizontal line indicates one patient. The vertical bar on the left of the heatmap indicates the occurrence of plasma leakage in the patient. Cluster A had the highest proportion of patients with plasma leakage. Cluster B contained the majority of the healthy controls. Cluster C consisted of mainly DENV infected patients and had a lower proportion of patients with plasma leakage than cluster A.

5. Discussion

In this study, we have shown increased levels of Ang-2, MMP-2 and ET-1 and decreased levels of sVEGFR-2 in patients with plasma leakage. Moreover, cluster analysis confirmed that increased expression of Ang-2 and decreased expression of sVEGFR-2 was strongly associated with plasma leakage.

Patients with plasma leakage were significantly younger than patients without plasma leakage, which is in line with epidemiological studies that have shown higher frequencies of plasma leakage and a propensity to develop dengue shock syndrome in children relative to adults [31–33]. It has been hypothesized that the capillaries in growing children are more fragile and more sensitive to vasoactive agents than the capillaries of adults [34].

In this study, patients were most frequently included on day 5 after the onset of fever. It has been shown that DENV infected patients are viraemic for five days on average [35]. Moreover, patients with secondary DENV infection showed higher viral titres, but a shorter duration of their viraemia than patients with primary DENV infection [35]. This may explain why patients with plasma leakage in our study had a lower viral load and were more often experiencing a secondary infection compared to patients without plasma leakage.

This is the first time that increased levels of ET-1 have been detected in DENV infected patients with plasma leakage. ET-1 was first isolated from porcine aortic endothelial cells and is known to be a potent inducer of vasoconstriction [18]. In patients with septic shock, elevated levels of ET-1 were associated with disease severity and correlated significantly with a lower cardiac index, most probably due to suppression of the cardiac function by ET-1 [20]. Interestingly, a low cardiac index was also reported in patients with dengue shock syndrome [36].

MMPs can lyse the subendothelial basement membrane and could therefore contribute to the development of hyperpermeability. MMP-2 was significantly elevated in patients with plasma leakage compared to patients without plasma leakage, while MMP-9 was significantly elevated in DENV infected patients compared to healthy controls. It has been shown that DENV infection of microvascular endothelial cells induced secretion of MMP-2 more strongly than secretion of MMP-9 [37]. In contrast, DENV infected dendritic cells secreted higher levels of MMP-9 than MMP-2 [38]. The association between MMP-2 (but not MMP-9) and the occurrence of plasma leakage suggests that endothelial cell activation plays an important role in the development of vascular leakage.

No significant differences in VEGF levels in patients with and without plasma leakage were detected. Some studies have reported increased levels of plasma VEGF in severe versus uncomplicated dengue [8,9,39], while others did not detect any differences in levels between severity groups [40,41]. Both in the supervised clinical classification and in the unsupervised cluster analysis, Ang-2 and sVEGFR-2 were strongly associated with the occurrence of plasma leakage, which is in line with other clinical studies [8,16]. In vitro studies also showed that DENV infection of endothelial cells resulted in decreased levels of sVEGFR-2 and increased Ang-2 expression [8,42]. Ang-2 is synthesized by endothelial cells and quickly released in the circulation upon activation [43]. Ang-1 and Ang-2 are antagonistic ligands of the Tie-2 receptor, a vascular specific tyrosine kinase receptor. Ang-1/Tie-2 signalling brings the endothelium in a quiescent state, while Ang-2/Tie-2 signalling results in endothelial detachment [15]. In line with this study, increased levels of circulating endothelial cells have been detected in patients with DHF [44]. Another study showed that Ang-2 stimulation of endothelial cells resulted in an increase in vascular permeability due to downregulation of proteins in the adherent and tight junctions between the cells [42]. Interestingly, VEGF-sVEGFR-2-signalling also affects the adherens junction, because it causes endocytosis of VE-cadherin in the endothelial cell [45].

Table 2
Characteristics of the three clusters in the cluster analysis.

	Cluster A ($N=60$)	Cluster B ($N=32$)	Cluster C ($N=27$)	Statistics
Age*	15 (11–46)	11 (9–17)	18 (9–33)	$p=0.6$ (KW)
Gender	65% ($N=39$) male	53% ($N=17$) male	63% ($N=17$) male	$p=0.53$ (Chi)
2009 WHO dengue case classification	20% ($N=12$) WS− 75% ($N=45$) WS+ 2% ($N=1$) Severe 3% ($N=2$) HC	31% ($N=10$) WS− 34% ($N=11$) WS+ 34% ($N=11$) HC	52% ($N=14$) WS− 44% ($N=12$) WS+ 4% ($N=1$) HC	$p<0.0001$ (Chi)
Plasma leakage	62% ($N=37$)	19% ($N=6$)	22% ($N=6$)	$p<0.0001$ (Chi)
Haemorrhage	42% ($N=25$)	13% ($N=4$)	41% ($N=11$)	$p<0.0001$ (Chi)
Angiopoietin-2* (pg/ml)	20.751 (14.851–27.775) MV = 2	8.806 (6.463–12.524) MV = 1	8.210 (6.926–9.703) MV = 1	NA
Endoglin* (ng/ml)	13.5 (11.5–15.2)	13.4 (10.2–16.0)	14.1 (12.2–17.1)	NA
Endothelin-1* (pg/ml)	10.4 (8.2–12.8) MV = 1	8.2 (6.8–11.4)	7.8 (5.5–9.6)	NA
MMP-2* (ng/ml)	756 (645–851) MV = 1	685 (587–779)	589 (539–696)	NA
MMP-9* (ng/ml)	470 (297–648) MV = 1	170 (115–253) MV = 1	982 (721–1511) MV = 1	NA
sVEGFR-2* (pg/ml)	7.893 (6.394–10.168) MV = 3	10.769 (9.525–18.341)	18.277 (13.342–25.234) MV = 1	NA
TIMP-1* (ng/ml)	874 (641–1137)	529 (397–986) MV = 1	861 (625–1199) MV = 1	NA
TIMP-2* (ng/ml)	302 (233–354)	291 (260–347)	275 (207–304)	NA

Abbreviations: MV = missing value (due to failure in the assay, e.g. variation in duplo too large). KW = Kruskal Wallis test. Chi = Chi-squared test. WS− = non-severe dengue without warning signs. WS+ = non-severe dengue with warning signs. HC = healthy control. NA = not applicable, e.g. it is not permitted to perform statistics on values that determine the formation of the clusters.

* Values are in median (interquartile range).

Because of the cross-sectional study design we could not draw any conclusions about the predictive value of Ang-2 and sVEGFR-2 before the onset of plasma leakage. However, different analysis techniques all indicated that Ang-2 and sVEGFR-2 are strongly associated with the presence of plasma leakage and can therefore be considered as surrogate markers.

Patients with plasma leakage had significantly lower levels of platelets than those without plasma leakage. Moreover, the platelet count showed a strong correlation with Ang-2 and sVEGFR-2. Thrombocytopenia is one of the hallmarks of a DENV infection and has been shown to correlate inversely with the occurrence of plasma leakage [46]. Besides the low number, it is hypothesized

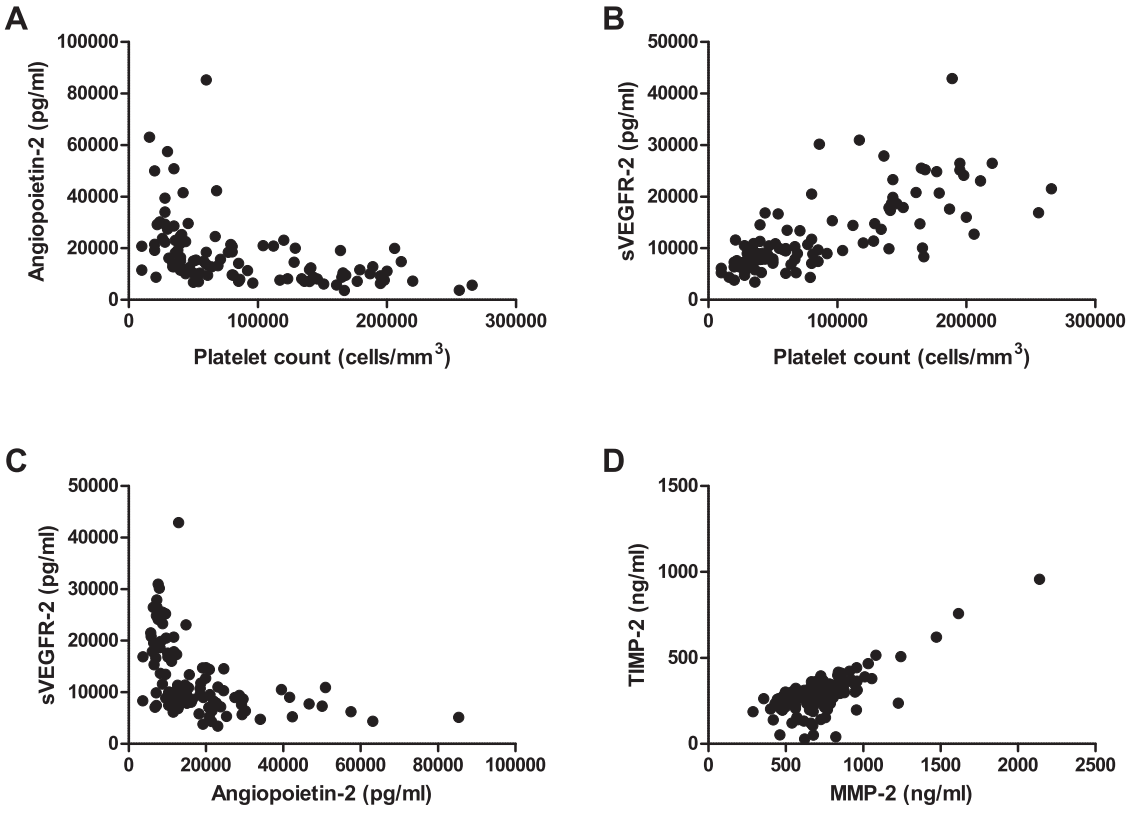

Fig. 4. Angiopoietin-2 and sVEGFR-2 are correlated with the platelet count and each other. (A) Ang-2 shows a significant inverse correlation with the platelet count ($\rho=-0.63$ ($p<0.0001$). (B) sVEGFR-2 shows a significant direct correlation with the platelet count ($\rho=0.74$ ($p<0.0001$)). (C) Ang-2 showed a significant inverse correlation with sVEGFR-2 ($\rho=-0.52$ ($p<0.0001$)). (D) MMP-2 shows a significant direct correlation with TIMP-2 ($\rho=0.60$ ($p<0.0001$)). Missing values: platelet count ($N=2$), Ang-2 ($N=2$), sVEGFR-2 ($N=2$), MMP-2 ($N=1$).

that the function of platelets is also impaired in DENV infected patients [46]. Under normal circumstances, platelets play a crucial role in stabilizing the vascular barrier and therefore, the presence of thrombocytopenia and thrombocytopathy, may contribute highly to endothelial cell dysfunction (reviewed in Ref. [47]).

In summary, we conclude that Ang-2 and sVEGFR-2 showed a strong correlation with the occurrence of plasma leakage in DENV infected patients. It is important to note that these markers did not suffer interference by the days after the onset of symptoms, suggesting they could serve as surrogate markers for plasma leakage in patients with acute DENV infection.

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The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing interests

None of the listed authors declare conflict of interest apart from Albert Osterhaus who is a part time employee of Viroclinics BV (for details go to www.erasmusmc.nl). The stated competing interest does not alter the author's adherence to the policies on sharing data and materials.

Ethical approval

All procedures adopted in this study were performed according to the terms agreed by the Institutional Review Board from Hospital das Clínicas, University of São Paulo (CAPPesq – Research Projects Ethics Committee). This study was approved by CAPPesq under protocol 0652/09. Written informed consent was obtained from all study volunteers. All included study participants were deidentified and a study number was assigned to guarantee confidentiality.

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Appendix A. Supplementary data

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